

A new angiogenic factor and its inhibitors

5 The present invention relates to a new angiogenic factor and its use in pharmaceutical and diagnostic compositions. Furthermore, the invention relates to inhibitors of the factor and their pharmaceutical use.

10 Angiogenesis, the growth of new capillaries from pre-existing ones, is critical for normal physiological functions in adults [Carmeliet, P., Mechanisms of angiogenesis and arteriogenesis. Nat Med, 2000 6 (4) 389-95]. Abnormal angiogenesis can lead to impaired wound healing, poor tissue regeneration in ischemic conditions, cyclical growth of the female reproductive system, and tumor development [Carmeliet, P. and R. K. Jain, Angiogenesis in cancer and other diseases. Nature,
15 2000 407: 249-257].

Promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial
20 stenosis. The angiogenic process is highly complex and involves the maintenance of the endothelial cells in the cell cycle, degradation of the extracellular matrix, migration and invasion of the surrounding tissue and finally, tube formation. Because of the crucial role of angiogenesis in so many physiological processes, there is a need to identify and characterize factors which will promote angiogenesis.

25

The administration of growth factors such as VEGF-A and FGF-2 has been considered as a possible approach for the therapeutic treatment of ischemic disorders.

- 2 -

VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer that is released by a variety of tumor cells and expressed in human tumor cells in situ.

However, both animal studies and early clinical trials with VEGF angiogenesis
5 have encountered severe problems [Carmeliet, Nat Med, 2000 6 1102-3; Yancopoulos et al., Nature, 2000 407 242-8; Veikkola et al., Semin Cancer Biol 1999 9 211-20; Dvorak et al., Semin Perinatol 2000 24 75-8; Lee et al., Circulation, 2000 102 898-901]. VEGF-A stimulated microvessels are disorganized, sinusoidal and dilated, much like those found in tumors [Lee et al., Circulation 2000
10 102 898-901; and Springer et al., Mol. Cell 1998 2 549-559]. Moreover, these vessels are usually leaky, poorly perfused, torturous and likely to rupture and regress. Thus, these vessels have limited ability to improve the ischemic conditions. In addition, the leakage of blood vessels induced by VEGF-A (also known as Vascular Permeability Factor) could cause cardiac edema that leads to heart fail-
15 ure.

VEGF not only stimulates vascular endothelial cell proliferation, but also induces vascular permeability and angiogenesis. Angiogenesis, which involves the formation of new blood vessels from preexisting endothelium, is an important component of a variety of diseases and disorders including tumor growth and metastasis,
20 rheumatoid arthritis, psoriasis, atherosclerosis, retinopathy, hemangiomas, immune rejection of transplanted tissues, and chronic inflammation.

In the case of tumor growth, angiogenesis appears to be crucial for the transition
25 from hyperplasia to neoplasia, and for providing nourishment to the growing solid tumor. [Folkman, et al., Nature 339:58 (1989)]. Angiogenesis also allows tumors to be in contact with the vascular bed of the host, which may provide a route for metastasis of the tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies showing a correlation between the
30 number and density of microvessels in histologic sections of invasive human

- 3 -

breast carcinoma and actual presence of distant metastases. [Weidner, et al., New Engl. J. Med. 324:1 (1991)].

5 Expression analyses, which are shown in figure 3, show the presence of significant levels of the well known pro-angiogenic factor VEGF in tumor tissues, reflecting the above described requirement for stimulation of vascular growth into tumors, particularly solid tumors. On the other hand, the expression levels of VEGF are clearly detectable not only in malignant tissues, but also in a variety of normal cells and tissues. Consequently, the concentration of VEGF is predicted to
10 be increased around the tissues which contain VEGF expression cells (Figure 3). This, in turn, may indicate the need not only of tumor tissue, but also of various normal tissues for VEGF mediated vascular growth. Therefore, VEGF is not a promising target when tumors, but not the surrounding tissue, are to be specifically attacked.

15 In summary, therapeutic agents promoting revascularization with minimal toxicity are still needed and there is an ongoing requirement for new angiogenic factors and new methods of angiogenic therapy. Furthermore, there is a need for factors which specifically inhibit neovascularization in solid tumors.

20 The problem underlying the present invention therefore lies in providing an angiogenic agent which does not exhibit the deficiencies of VEGF as depicted above.

25 In the context of the present invention, it has been surprisingly found that the human protein disclosed in the NCBI database entries BAA86585, AAH44952 (see SEQ ID NO: 4) and XP_045472 (see SEQ ID NO: 6) exhibits an important role in angiogenesis both in its membrane bound form (BAA86585, AAH44952 and XP_045472) as well as in a soluble form. This protein was named SEP, and its
30 soluble, not membrane bound form was named sSEP. The corresponding cDNA sequences of the membrane bound form are given in the NCBI database entries

- 4 -

BC044952 and XM_045472 (SEQ ID NO: 3 and 5). Therefore, the SEP and sSEP are a novel angiogenic factors of a to-date unknown novel family. The corresponding mouse sequences of SEP (mSEP) are given in SEQ ID NO: 1 (DNA) and 2 (protein).

5

Consequently, according to one aspect of the invention, the problem is solved by a soluble SEP (sSEP) or a functional active soluble derivative thereof.

In the context of the present invention, it could be demonstrated that SEP mediates strong angiogenic activity.

10

This result is totally surprising, since its sequence is not homologous to the sequence of VEGF. In Example 8, it is demonstrated that transfection of cells with DNA encoding SEP leads to the production of VEGF, and Example 17 shows that other angiogenic factors like IL-8 and RANTES are induced by SEP.

15

The term "sSEP" relates to any soluble SEP, wherein the amino acid sequence of SEP as demonstrated in the database has been manipulated with the consequence that the manipulated protein is soluble. In this context, sSEP relates both to artificial as well as to naturally occurring proteins.

20

In a preferred embodiment of the invention, the sSEP of the invention does not comprise a transmembrane domain. According to Fig. 4, the transmembrane domain of SEP extends at least from amino acid 514 to amino acid 535 of the human SEP as disclosed in the data base entries AAH44952 (see SEQ ID NO: 4) and XP_045472 (see SEQ ID NO: 6). An sSEP can therefore be produced by changing the amino acid sequence in this putative transmembrane region, e.g. by exchanging hydrophobic amino acids with hydrophilic amino acids.

25

Example 9 clearly demonstrates that sSEP has angiogenic properties.

30

- 5 -

Methods for the production of proteins starting from a cDNA are known in the art and include e.g. the expression of the protein in appropriate cells or the production by subsequent addition of amino acids to a starting amino acid (Current Protocols, John Wiley & Sons, Inc., New York (2003)).

5

Furthermore, methods for the production of protein fragments are known in the art and include the cleavage of the protein with appropriate proteases or the generation of nucleic acid fragments encoding the protein fragments and subsequent expression of the fragments in appropriate cells (Current Protocols, John Wiley & Sons, Inc., New York (2003)).

10

Methods for the production of mutated proteins and therefore of sSEP, e.g. by exchanging one or more amino acids or by deleting stretches of amino acids, are known in the art. These methods include site directed mutagenesis of the SEP gene e.g. as disclosed in the database entries BC 044952 and XM_045472, and expressing the modified gene in appropriate cells (Current Protocols, John Wiley & Sons, Inc., New York (2003)).

15

The term "functional active derivative" of a polypeptide within the meaning of the present invention refers to polypeptides which have a sequence homology, in particular a sequence identity, of about at least 25 %, preferably about 40 %, in particular about 60 %, especially about 70 %, even more preferred about 80 %, in particular about 90 % and most preferred of 98 % with the polypeptide, which has essentially the biological function(s) as the corresponding protein. In the case of SEP or sSEP, this may be an angiogenic activity as demonstrated in Examples 2 and 3. A test for the determination of the angiogenic activity of a putative sSEP derivative is demonstrated in Example 2.

25

Such derivatives are e.g. the polypeptide homologous to sSEP or SEP, which originate from organisms other than human. Other examples of derivatives are polypeptides which are encoded by different alleles of the gene, of different indi-

30

- 6 -

viduals, in different organs of an organism or in different developmental phases. Functional active derivatives preferably also include naturally occurring mutations, particularly mutations that quantitatively alter the activity of the peptides encoded by these sequences. Further, such variants may preferably arise from differential splicing of the encoding genes.

The term "functional active soluble derivative" refers to a soluble, i.e. not membrane-bound, "functional active soluble derivative" as defined above.

10 In an especially preferred embodiment of the invention, the term "functional active derivative" or "functional active soluble derivative" include derivatives with single nucleotide polymorphism (SNP) at at least one of the positions 383 (G to C), 699 (A to C), 1332 (T to C), 1778 (C to T), 2260 (C to A) and/or 2896/7 (TT to GA) of the nucleotide sequence given in SEQ ID NO: 3 (BC044952).

15

Most preferred are SNPs at positions 383, 699 and/or 1332, leading to the amino acid exchanges E with Q, K with Q and F with S, respectively.

"Sequence identity" refers to the degree of identity (% identity) of two sequences, that in the case of polypeptides can be determined by means of for example BLASTP 2.2.5 and in the case of nucleic acids by means of for example BLASTN 2.2.6, wherein the low complexity filter is set on and BLOSUM is 62 (Altschul et al., 1997, Nucleic Acids Res., 25:3389-3402).

25 "Sequence homology" refers to the similarity (% positives) of two polypeptide sequences determined by means of for example BLASTP 2.0.1, wherein the filter is set on and BLOSUM is 62 (Altschul et al., 1997, Nucleic Acids Res., 25:3389-3402).

- 7 -

Nucleic acids encoding functional active derivatives can be isolated by using human SEP gene sequences in order to identify homologues with methods known to a person skilled in the art, e.g. through PCR amplification or hybridization under stringent conditions (e.g. 60 °C in 2.5 x SSC buffer followed by several washing
5 steps at room temperature) with suitable probes derived from e.g. the human SEP sequences according to standard laboratory methods.

Furthermore, in case of sSEP, the same biological activity may also be the ability to compete with membrane bound SEP and therefore to act as an inhibitor of a
10 signal transduced by membrane bound SEP.

In the case of membrane bound and soluble SEP, the term "functional active derivative" may refer to the ability to induce the expression of VEGF as shown in Example 8.

15 According to a preferred embodiment of the invention, the sSEP or functional derivative thereof of the invention is devoid of a transmembrane domain of SEP or of functional active variant thereof. Preferably, this means that a C-terminal fragment containing the transmembrane domain of the SEP or of the functional
20 active derivative thereof has been cleaved off. More preferably, also an N-terminal fragment has been cleaved off. Preferably, sSEP fragments are produced by cleaving at potential protease cleaving sites, more preferably at the following potential cleaving sites:

25 SPRAIPRN (amino acids 165 to 172 of SEP as given in SEQ ID NO: 4)
ARSTPRASRL (amino acids 242 to 250 of SEP as given in SEQ ID NO: 4)
HRPSP (amino acids 509 to 513 of SEP as given in SEQ ID NO: 4)

Cleaving can occur within every amino acid within these sequences, however, a
30 cleaving after the amino acid R is preferred.

- 8 -

According to the invention, this includes also that after cleavage with an appropriate protease, further amino acids are removed by e.g. carboxypeptidases.

Consequently, in a more preferred embodiment, the sSEP or functional derivative thereof of the invention has a C-terminal amino acid corresponding to amino acid 510, 249, 246, 242, 171 or 167 of SEP according to SEQ ID NO: 4 or has a C-terminal amino acid corresponding to the equivalent amino acid of a SEP derivative.

10 In a most preferred embodiment, an sSEP according to the invention has one of the sequences as shown in Figure 5 (SEQ ID NO: 7-18).

Within the invention it is also included that, in case that a fragment of the invention still comprises a signal peptide, this signal peptide may also be cleaved off.

15

As demonstrated first in the context of the present invention, the protein depicted in SEQ ID NO: 2, 4 or 6 and soluble variants thereof exhibit an important role in angiogenesis. This enables the use of these proteins in therapy.

20 Consequently, the invention further relates to a pharmaceutical composition comprising

- a) the sSEP or derivative thereof of the invention,
- b) SEP as defined in SEQ ID NO: 2, 4 or 6,
- 25 c) a functional active derivative of the SEP of section b), and/or
- d) a nucleic acid encoding the proteins of sections a), b) or c) above,

optionally in combination with a pharmaceutically acceptable carrier.

30 The molecules as depicted in sections a) to d) may be provided as defined above.

- 9 -

Examples of nucleic acids as defined in d) are the nucleic acids shown in SEQ ID NO: 1, 3, and 5. Other examples are nucleic acids encoding the derivatives and fragments as described above.

5 In a preferred embodiment of the invention, the pharmaceutical composition further comprises VEGF, and/or a functional derivative thereof, preferably in combination with VEGF-A, VEGF-B, VEGF-C, VEGF-D, PLGF, PDGF-A, PDGF-B, PDGF-C, PDGF-D and FGF.

10 As already mentioned above, VEGF is a well known angiogenic factor. A combination of SEP and VEGF leads to enforced or synergistic effects in the promotion of angiogenesis in mammals.

The invention also relates to the sSEP or derivative thereof of the invention or a
15 SEP as defined in SEQ ID NO: 2, 4 or 6 or functional active derivatives thereof or of nucleic acids encoding these molecules for use in therapy.

The pharmaceutical composition of the invention may be applied as follows:

20 In accordance with the invention, there are numerous techniques which can be used to administer an effective vasculogenesis promoting or angiogenesis stimulating amount of SEP, sSEP or a functional active derivative thereof to a patient suffering from ischemia or some other condition which may be alleviated by vasculogenesis or angiogenesis. SEP administration may be effected either as recombinant protein or by gene transfer either as naked DNA or in a vector [Kornowski
25 R, Fuchs S, Leon MB, Epstein SE, Delivery strategies to achieve therapeutic myocardial angiogenesis, Circulation, 2000 101 (4) 454-8; Simons M, Bonow RO, Chronos NA, Cohen DJ, Giordano FJ, Hammond HK, et al., Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary, Circulation, 2000 102 (11) E73-86; and Isner JM, Asahara T, Angiogenesis and
30

- 10 -

vasculogenesis as therapeutic strategies for postnatal neovascularization, *J Clin Invest*, 1999 103 (9) 1231-36].

If desired, regulatable vectors may be used as described in Ozawa et al, *Annu Rev Pharmacol. & Toxicol*, 2000 40 295-317. For example, SEP or sSEP can be administered by direct myocardial injection of naked plasmid DNA encoding SEP, sSEP or a functional active derivative thereof during surgery in patients with chronic myocardial ischemia following procedures outlined in Vale, P. R., et al., Left ventricular electromechanical mapping to assess efficacy of phVEGF (165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia, *Circulation*, 2000 102 965-74. SEP, sSEP or a functional active derivative thereof can also be administered by direct myocardial injection of SEP, sSEP or a functional active derivative thereof protein via a minithoracotomy. Preferably, it is given as a bolus dose of from 1 pg/kg to 15 mg/kg, preferably between 5 pg/kg and 5 mg/kg, and most preferably between 0.2 and 2 mg/kg. Continuous infusion may also be used, for example, by means of an osmotic minipump as described in Heyman et al., *Nat Med*, 1999 5 1135-152. If so, the medicament may be infused at a dose between 5 and 20 $\mu\text{g}/(\text{kg}\cdot\text{minute})$, preferably between 1 and 100 $\text{ng}/(\text{kg}\cdot\text{minute})$, more preferably between 5 and 20 $\text{ng}/(\text{kg}\cdot\text{minute})$ and most preferably between 7 and 15 $\text{pg}/(\text{kg}\cdot\text{minute})$.

Alternatively SEP, sSEP or a functional active derivative thereof can be administered by catheter-based myocardial gene transfer of SEP, sSEP or a functional active derivative thereof. In this technique, a steerable, deflectable 8F catheter incorporating a 27gauge needle is preferably used and advanced percutaneously to the left ventricular myocardium. For example, a total dose of 200 $\mu\text{g}/\text{kg}$ is administered as 6 injections into the ischemic myocardium (total, 6.0 ml). Injections are guided by e.g. NOGA left ventricular electromechanical mapping. See Vale, P. R., et al., Randomized, single-blind, placebo-controlled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular elec-

- 11 -

tromechanical mapping in patients with chronic myocardial ischemia, *Circulation*, 2001 103 (17) 2138-43.

Another possibility for SEP, sSEP or a functional active derivative thereof administration is injection of SEP plasmid in e.g. the muscles of an ischemic limb in
5 accordance with procedures described in Simovic, D., et al., Improvement in chronic ischemic neuropathy after intramuscular gene transfer e.g. using phVEGF165 in patients with critical limb ischemia, *Arch Neurol*, 2001 58 (5) 76168.

10

Still another technique for effective administration is by intra-arterial gene transfer of the gene using adenovirus and replication defective retroviruses as described for VEGF in Baumgartner I and Isner JM, Somatic gene therapy in the cardiovascular system, *Annu. Rev Physiol*, 2001 63 427-50. An additional possibility for administering SEP, sSEP or a functional active derivative thereof is by
15 intracoronary and intravenous administration of recombinant SEP, sSEP or a functional active derivative thereof following procedures described in Post, M. J., et al., Therapeutic angiogenesis in cardiology using protein formulations, *Cardio-vasc Res*, 2001 49 522-31.

20

A still further possibility is to use ex vivo expanded endothelial progenitor cells (EPCs) engineered to express SEP, sSEP or a functional active derivative thereof for myocardial neovascularization as described in Kawamoto, A., et al., Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial
25 ischemia. *Circulation*, 2001 103 (5) 634-37.

Yet another technique which may be used to administer SEP, sSEP or a functional active derivative thereof is percutaneous adenovirus-mediated gene delivery to the arterial wall in e.g. injured atheromatous stented arteries. See, for example, Mail-
30 lard, L., et al., Effect of percutaneous adenovirus-mediated Gax gene delivery to the arterial wall in double-injured atheromatous stented rabbit iliac arteries, *Gene*

- 12 -

Ther, 2000 7 (16) 1353-61 ; and Laham RJ, Simons M, and Sellke F, Gene transfer for angiogenesis in coronary artery disease, Annu Rev Med, 2001 52 485-502.

In one advantageous aspect of the invention, a therapeutically effective dose of
5 SEP, sSEP or a functional active derivative thereof is administered by bolus injection of the active substance into e.g. ischemic tissue, e. g. heart or peripheral muscle tissue. The effective dose will vary depending on the weight and condition of the ischemic subject and the nature of the ischemic condition to be treated. It is considered to be within the skill of the art to determine the appropriate dosage for
10 a given subject and condition. Furthermore, the pharmaceutical composition can be administered in further conventional manners, e.g. by means of the mucous membranes, for example the nose or the oral cavity, in the form of dispositories, implanted under the skin, by means of injections, infusions or gels which contain the medicaments according to the invention. It is further possible to administer the
15 medicament topically and locally, if appropriate, in the form of liposome complexes. Furthermore, the treatment can be carried out by means of a transdermal therapeutic system (TTS), which makes possible a temporally controlled release of the medicaments. TTS are known for example, from EP 0 944 398 A1, EP 0 916 336 A1, EP 0 889 723 A1 or EP 0 852 493 A1.

20

In accordance with another aspect of the invention, SEP, sSEP or a functional active derivative thereof is administered by continuous delivery, e. g., using an osmotic minipump, until the patient is able to self maintain a functional vascular network.

25

In another advantageous aspect within the scope of the invention, SEP, sSEP or a functional active derivative thereof is effectively administered to an ischemic subject by contacting ischemic tissue with a viral vector, e. g. an adenovirus vector, containing a polynucleotide sequence encoding the protein operatively linked to a
30 promoter sequence.

- 13 -

SEP, sSEP or a functional active derivative thereof may also be effectively administered by implantation of a micropellet impregnated with active substance in the direct vicinity of e.g. the ischemic tissue.

- 5 For the production of the pharmaceutical compositions of the invention, the molecules of the present invention are usually formulated with suitable additives or auxiliary substances, such as physiological buffer solution, e.g. sodium chloride solution, demineralized water, stabilizers, such as protease or nuclease inhibitors, preferably aprotinin, ϵ -aminocaproic acid or pepstatin A or sequestering agents
10 such as EDTA, gel formulations, such as white Vaseline, low-viscosity paraffin and/or yellow wax, etc. depending on the kind of administration.

Suitable further additives are, for example, detergents, such as, for example, Triton X-100 or sodium deoxycholate, but also polyols, such as, for example, poly-
15 ethylene glycol or glycerol, sugars, such as, for example, sucrose or glucose, zwitterionic compounds, such as, for example, amino acids such as glycine or in particular taurine or betaine and/or a protein, such as, for example, bovine or human serum albumin. Detergents, polyols and/or zwitterionic compounds are preferred.

- 20 The physiological buffer solution preferably has a pH of approx. 6.0-8.0, especially a pH of approx. 6.8-7.8, in particular a pH of approx. 7.4, and/or an osmolarity of approx. 200-400 mosmol/l, preferably of approx. 290-310 mosmol/lr. The pH of the medicament is in general adjusted using a suitable organic or inorganic buffer, such as, for example, preferably using a phosphate buffer, tris buffer
25 (tris(hydroxymethyl)aminomethane), HEPES buffer ([4-(2-hydroxyethyl)piperazino]ethanesulphonic acid) or MOPS buffer (3-morpholino-1-propanesulphonic acid). The choice of the respective buffer in general depends on the desired buffer molarity. Phosphate buffer is suitable, for example, for injection and infusion solutions.

- 14 -

Injection solutions are in general used if only relatively small amounts of a solution or suspension, for example about 1 to about 20 ml, are to be administered to the body. Infusion solutions are in general used if a larger amount of a solution or suspension, for example one or more liters, are to be administered. Since, in contrast to the infusion solution, only a few milliliters are administered in the case of injection solutions, small differences from the pH and from the osmotic pressure of the blood or the tissue fluid in the injection do not make themselves noticeable or only make themselves noticeable to an insignificant extent with respect to pain sensation. Dilution of the formulation according to the invention before use is therefore in general not necessary. In the case of the administration of relatively large amounts, however, the formulation according to the invention should be diluted briefly before administration to such an extent that an at least approximately isotonic solution is obtained. An example of an isotonic solution is a 0.9% strength sodium chloride solution. In the case of infusion, the dilution can be carried out, for example, using sterile water while the administration can be carried out, for example, via a so-called bypass.

Within the present invention, subjects which may be treated or diagnosed include animals, preferably mammals and humans, dead or alive. These patients suffer from the diseases as mentioned above.

Furthermore, the invention relates to the use of

- a) the sSEP or derivative thereof of the invention,
- b) SEP as defined in SEQ ID NO: 2, 4 or 6,
- c) a functional active derivative of the SEP of section b), and/or
- d) a nucleic acid encoding the molecules of sections a), b) or c)

for the preparation of a pharmaceutical composition for the treatment of ischemic, dental or placental diseases, of smoker's leg and diabetic ulcers, for the stimulation of wound healing, especially of wound healing of fractures, or for the amelioration or preservation of infertility. With respect to this use of the invention and

- 15 -

especially with respect to the administration, the dosage and the manufacture of this pharmaceutical composition, the same applies as defined above.

5 These diseases are all characterized by a disturbed angiogenesis and therefore SEP, either as a soluble factor or as defined in SEQ ID NO: 2, 4 or 6 as well as functional active derivatives thereof lead to a significant improvement in these diseases.

10 With respect to the wound healing of fractures, SEP immobilized to a matrix can be administered directly into the site of fracture to promote the angiogenesis and wound healing. As matrices can be used ceramic matrices or bonemeal on which the protein is immobilized. Slow release formulations to have the factor locally enriched can be used as well.

15 With respect to the treatment of placental diseases, neovascularization is an essential requirement for supporting the growing fetus and embryo during pregnancy. For that process, vascular development is necessary in the placenta (fetal as well as maternal tissue) as well as in the uterus. Expression analyses, which are shown in Figure 3, show the presence of significant levels of VEGF in uterus, reflecting
20 the above described requirement for stimulation of vascular growth in this tissue. On the other hand, compared with placenta, the expression levels of VEGF are relatively low in placenta. Thus, the limited expression of VEGF in placenta may - by itself - not be sufficient to stimulate sufficient vascularization. The high expression of SEP in female placenta, as shown in figure 3, provides an explanation
25 for the lower levels of VEGF expression in placenta compared to uterus. SEP is highly expressed in normal placenta. Hereby, both factors, each with defined specificity, are complementing their function to stimulate vascularization. In consequence, both factors are necessary for sufficient vascularization during pregnancy.

- 16 -

Because of that, deficiencies in SEP may cause infertility, problems in pregnancy. Consequently, supplementation of SEP may aid to ameliorate or prevent said disorders. Furthermore, inhibition of SEP may be used to prevent angiogenesis in early pregnancies, with the objective to terminate pregnancies in humans (or animals) due to medical indications.

As explained above, SEP is a strong angiogenic factor. Therefore, in a preferred embodiment of the use of the present invention, the molecules as defined in sections a) to d) induce the formation of vascular vessels.

As it can be taken from Example 8, sSEP, SEP or the functional active derivative thereof are able to induce the production of VEGF. Therefore, in a preferred embodiment of the use of the present invention, the molecules as defined in sections a) to d) (as defined above) induce the production of VEGF.

In a further preferred embodiment, the molecules as defined in sections a) to d) (as defined above) induce the production of IL-8 and/or RANTES.

In a preferred embodiment of the invention, sSEP, SEP or functional active derivatives thereof are used in combination with VEGF and/or functional active derivatives thereof, preferably in combination with VEGF-A, VEGF-B, VEGF-C, VEGF-D, PLGF, PDGF-A, PDGF-B, PDGF-C, PDGF-D and FGF.

The invention further includes a method for the treatment of a patient in need of such treatment, wherein an effective amount of SEP, sSEP or a functional active derivative thereof is administered to the patient.

With respect to the preparation of this pharmaceutical composition, its administration and other embodiments, the same applies as defined above.

30

- 17 -

Another subject of the present invention is an antibody or fragment thereof which specifically binds an sSEP or derivative thereof of the invention, SEP as defined in SEQ ID NO: 2, 4 or 6 or a functional active derivative of the SEP of the invention. In a preferred embodiment the antibody is a monoclonal or polyclonal antibody. The procedure for preparing an antibody or antibody fragment as described in Example 18 or 20 is effected in accordance with methods which are well known to the skilled person (see below). The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity. "Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

As it is shown in Examples 7 and 10 to 14, SEP is especially upregulated in several tumor diseases. Consequently, SEP, sSEP and functional active derivatives thereof can be used as diagnostic agents.

The invention therefore relates to a diagnostic agent comprising

- a) the sSEP or derivative thereof of the invention
- b) SEP as defined in SEQ ID NO: 2, 4 or 6,
- c) a functional active derivative of the SEP of section b),
- d) a nucleic acid encoding the SEPs of sections a), b) or c), and/or
- e) means for detection of the molecules of sections a), b), c) or d).

This diagnostic agent may be appropriately combined with additional carriers or diluents or other additives which are suitable in this context. With respect to these

- 18 -

agents, the same apply as defined above for the pharmaceutical composition of the invention.

Furthermore, the invention relates to the sSEP or derivatives thereof of the invention, SEP as defined in SEQ ID NO: 2, 4 or 6, a functional active derivative thereof, a nucleic acid encoding these SEPs or functional active derivatives and/or of means for detecting these SEPs or nucleic acids for use in therapy or diagnosis.

The proteins or nucleic acids may be prepared as defined above.

10

Within the meaning of the present invention, means of detecting the proteins of the invention or SEP or functional active derivatives thereof include antibodies or fragments thereof which specifically bind an sSEP or derivative thereof of the invention, SEP as defined in SEQ ID NO: 2, 4 or 6 or a functional active derivative of the SEP. The antibody or fragment thereof can be e.g. a monoclonal or polyclonal antibodies or fragments thereof. It can e.g. be applied in Western Blotting, Immunohistochemistry, ELISA or functional assays for the proteins (Current Protocols, John Wiley & Sons, Inc. (2003)).

15

20 Means for detecting the nucleic acids as defined above include other nucleic acids being capable of hybridizing with the nucleic acids e.g. in Southern Blots or Northern Blots as well as during In Situ Hybridization (Current Protocols, John Wiley & Sons, Inc. (2003)).

25

Furthermore, the invention relates to the use of

- a) the sSEP or derivatives of the invention,
 - b) SEP as defined in SEQ ID NO: 2, 4 or 6,
 - c) a functional active derivative of the SEP of section b),
 - d) a nucleic acid encoding the molecules of sections a, b, or c) and/or
 - 30 e) means for detection of the molecules of sections a), b), c) or d)
- for the diagnosis of tumor or tumor progression.

- 19 -

SEP is an important marker of tumor cells (as shown in Fig. 3). Angiogenesis is generally a phenomenon which occurs in later tumor stages. Therefore, SEP represents a marker for later tumor stages, i.e. for tumors which have already
5 achieved a malignant state.

For example, sSEP or functional active derivatives thereof may be detected in the serum via antibodies. Furthermore, SEP, sSEP or functional active derivatives thereof may be detected in the tumor tissue via immunohistochemistry. Nucleic
10 acids encoding these molecules, e.g. mRNA, may be detected using quantitative PCR.

Depending of the tumor progression and of the occurrence of a tumor, sSEP expression in the serum may change. Consequently, by measuring serum levels, it
15 can be determined whether a patient is susceptible for an SEP or sSEP mediated tumor therapy. The higher the SEP or sSEP expression, the better a therapeutical success can be predicted.

In several diseases as mentioned below, an aberrant angiogenesis contributes the
20 clinical symptoms or is even the reason for these symptoms. The present invention relates to SEP, which is an important inducer of angiogenesis, e.g. in tumors. In contrast to VEGF, the expression of SEP is predominantly restricted to tumor cells. Especially the expression of SEP in uterus appears to fulfill a defined biological function, as described further in Figure 3. The rather specific expression of
25 SEP in cancerous tissues makes SEP a valuable target for cancer therapy. Consequently, the inhibition of SEP results in inhibition of angiogenesis which will result in the treatment of these diseases. Because of the greater tumor-vs-normal specificity of SEP, said inhibitory substances have increased tumor specificity.

- 20 -

In another aspect of the invention, the invention therefore also relates to an inhibitor of the sSEP or derivatives thereof of the invention or of the SEP as defined in SEQ ID NO: 2, 4 or 6 or of functional active derivatives thereof.

5 According to the present invention the term "inhibitor" refers to a biochemical or chemical compound which preferably inhibits or reduces the angiogenic activity of sSEP, SEP or the derivatives thereof. This can e.g. occur via suppression of the expression of the corresponding gene. The expression of the gene can be measured by RT-PCR or Western blot analysis.

10

Examples of such SEP inhibitors are binding proteins or binding peptides directed against SEP, in particular against the active site of SEP, and nucleic acids directed against the SEP gene.

15 In a preferred embodiment, the inhibitor of the invention is selected from the group consisting of antibodies, peptides, SEP fragments, antisense oligonucleotides, siRNA, low molecular weight molecules (LMWs) and SEP receptor antagonists.

20 LMWs are molecules which are not proteins, peptides, antibodies or nucleic acids, and which exhibit a molecular weight of less than 5000 Da, preferably less than 2000 Da, more preferably less than 1000 Da, and most preferably less than 500 Da. Such LMWs may be identified in High-Through-Put procedures starting from libraries. Such methods are known in the art.

25

The term "binding protein" or "binding peptide" refers to a class of proteins or peptides which bind and inhibit sSEP, SEP or derivatives thereof including, without limitation, polyclonal or monoclonal antibodies, antibody fragments and protein scaffolds directed against these proteins.

30

- 21 -

In a preferred embodiment of the present invention the inhibitor of the present invention is an antibody or fragment thereof, preferably a polyclonal or monoclonal antibody. The procedure for preparing an antibody or antibody fragment as described in Example 18 or 20 is effected in accordance with methods which are well known to the skilled person, e.g. by immunizing a mammal, for example a rabbit, with sSEP, SEP or derivatives thereof, where appropriate in the presence of, for example, Freund's adjuvant and/or aluminum hydroxide gels (see, for example, Diamond, B.A. et al. (1981) *The New England Journal of Medicine*: 1344-1349) (see also Examples 18 and 19). The polyclonal antibodies which are formed in the animal as a result of an immunological reaction can subsequently be isolated from the blood using well known methods and, for example, purified by means of column chromatography. Monoclonal antibodies can, for example, be prepared in accordance with the known method of Winter & Milstein (Winter, G. & Milstein, C. (1991) *Nature*, 349, 293-299).

According to the present invention the term antibody or antibody fragment is also understood as meaning antibodies or antigen-binding parts thereof, which have been prepared recombinantly and, where appropriate, modified, such as chimeric antibodies, humanized antibodies, multifunctional antibodies, bispecific or oligospecific antibodies, single-stranded antibodies and F(ab) or F(ab)₂ fragments (see, for example, EP-B1-0 368 684, US 4,816,567, US 4,816,397, WO 88/01649, WO 93/06213 or WO 98/24884).

As an alternative to the classical antibodies it is also possible, for example, to use protein scaffolds against sSEP, SEP or derivatives thereof, e.g. anticalins which are based on lipocalin (Beste et al. (1999) *Proc. Natl. Acad. Sci. USA*, 96, 1898-1903). The natural ligand-binding sites of the lipocalins, for example the retinol-binding protein or the bilin-binding protein, can be altered, for example by means of a "combinatorial protein design" approach, in such a way that they bind to selected haptens, here to sSEP, SEP or derivatives thereof (Skerra, 2000, *Biochim. Biophys. Acta*, 1482, 337-50). Other known protein scaffolds are known as being

- 22 -

alternatives to antibodies for molecular recognition (Skerra (2000) J. Mol. Recognit., 13, 167-187).

5 If it is intended to inhibit the functions of membrane bound SEP, also sSEP may be an inhibitor of the invention, since sSEP may compete with SEP for the binding of SEP to an upstream or downstream component in its signal transduction cascade, e.g. its ligand or effector.

10 In another preferred embodiment of the invention the inhibitor of the present invention is a fragment of SEP. It was shown that fragments of SEP inhibit the SEP-mediated effects (see Example 21; reduction of SEP induced cell growth). In a more preferred embodiment the peptide comprises an amino acid sequence which encompasses at least 5, more preferably at least 7, amino acids and which occurs at least twice within the SEP sequence. In a still more preferred embodiment, the
15 peptide has the sequence as shown in SEQ ID NO: 26 or 27, in particular it has one of the sequences LPSKLPT (SEQ ID NO. 28), LPSKVPT (SEQ ID NO. 29), VPSKLPT (SEQ ID NO. 30), VPSKVPT (SEQ ID NO. 31) or LPSKLPT (SEQ ID NO. 27) (N- to C-terminal). Even more preferably a mixture of at least two, more preferably at least three, most preferably at least four of these peptides, e.g.
20 the four alternatives of SEQ ID NO: 26, is used.

The term "nucleic acids against the SEP gene or SEP itself" refers to double-stranded or single stranded DNA or RNA which, for example, inhibit the expression of the SEP gene or the activity of sSEP, SEP or derivatives thereof and includes, without limitation, antisense nucleic acids, aptamers, siRNAs (small interfering RNAs) and ribozymes.
25

The nucleic acids, e.g. the antisense nucleic acids or siRNAs, can be synthesized chemically, e.g. in accordance with the phosphotriester method (see, for example,
30 Uhlmann, E. & Peyman, A. (1990) Chemical Reviews, 90, 543-584). Aptamers are nucleic acids which bind with high affinity to a polypeptide, here sSEP, SEP

- 23 -

or derivatives thereof. Aptamers can be isolated by selection methods such as SELEX (see e.g. Jayasena (1999) Clin. Chem., 45, 1628-50; Klug and Famulok (1994) M. Mol. Biol. Rep., 20, 97-107; US 5,582,981) from a large pool of different single-stranded RNA molecules. Aptamers can also be synthesized and selected in their mirror-image form, for example as the L-ribonucleotide (Nolte et al. (1996) Nat. Biotechnol., 14, 1116-9; Klussmann et al. (1996) Nat. Biotechnol., 14, 1112-5). Forms which have been isolated in this way enjoy the advantage that they are not degraded by naturally occurring ribonucleases and, therefore, possess greater stability.

10

Nucleic acids may be degraded by endonucleases or exonucleases, in particular by DNases and RNases which can be found in the cell. It is, therefore, advantageous to modify the nucleic acids in order to stabilize them against degradation, thereby ensuring that a high concentration of the nucleic acid is maintained in the cell over a long period of time (Beigelman et al. (1995) Nucleic Acids Res. 23:3989-94; WO 95/11910; WO 98/37240; WO 97/29116). Typically, such a stabilization can be obtained by introducing one or more internucleotide phosphorus groups or by introducing one or more non-phosphorus internucleotides.

20 Suitable modified internucleotides are compiled in Uhlmann and Peyman (1990), supra (see also Beigelman et al. (1995) Nucleic Acids Res. 23:3989-94; WO 95/11910; WO 98/37240; WO 97/29116). Modified internucleotide phosphate radicals and/or non-phosphorus bridges in a nucleic acid which can be employed in one of the uses according to the invention contain, for example, methyl
25 phosphonate, phosphorothioate, phosphoramidate, phosphorodithioate and/or phosphate esters, whereas non-phosphorus internucleotide analogues contain, for example, siloxane bridges, carbonate bridges, carboxymethyl esters, acetamidate bridges and/or thioether bridges. It is also the intention that this modification should improve the durability of a pharmaceutical composition which can be employed in one of the uses according to the invention.
30

- 24 -

The use of suitable antisense nucleic acids is further described e.g. in Zheng and Kemeny (1995) Clin. Exp. Immunol., 100, 380-2; Nellen and Lichtenstein (1993) Trends Biochem. Sci., 18, 419-23, Stein (1992) Leukemia, 6, 697-74 or Yacyshyn, B. R. et al. (1998) Gastroenterology, 114, 1142).

5

The production and use of siRNAs as tools for RNA interference in the process to down regulate or to switch off gene expression, here SEP gene expression, is e.g. described in Elbashir, S. M. et al. (2001) Genes Dev., 15, 188 or Elbashir, S. M. et al. (2001) Nature, 411, 494. Preferably, siRNAs exhibit a length of less than 30 nucleotides, wherein the identity stretch of the sense strand of the siRNA is preferably at least 19 nucleotides.

10

Ribozymes are also suitable tools to inhibit the translation of nucleic acids, here the SEP gene, because they are able to specifically bind and cut the mRNAs. They are e.g. described in Amarzguioui et al. (1998) Cell. Mol. Life Sci., 54, 1175-202; Vaish et al. (1998) Nucleic Acids Res., 26, 5237-42; Persidis (1997) Nat. Biotechnol., 15, 921-2 or Couture and Stinchcomb (1996) Trends Genet., 12, 510-5.

15

Thus, the nucleic acids described can be used to inhibit or reduce the expression of the SEP genes in the cells both in vivo and in vitro and consequently act as a SEP inhibitor in the sense of the present invention. A single-stranded DNA or RNA is preferred for the use as an antisense oligonucleotide or ribozyme, respectively.

20

The invention further relates to a pharmaceutical composition, comprising the inhibitor of the invention, optionally in combination with a pharmaceutically acceptable carrier. With respect to the preparation and administration of this pharmaceutical composition of the invention, the same applies as defined above for other pharmaceutical compositions of the invention.

25

30

- 25 -

In a preferred embodiment, this pharmaceutical composition of the invention further comprises a VEGF inhibitor.

Another aspect of the invention relates to the inhibitor of the invention for use in
5 therapy.

The invention further relates to the use of an inhibitor of the invention for the preparation of a pharmaceutical composition for the treatment of cancer, rheumatoid arthritis, psoriasis, arteriosclerosis, retinopathy, osteoarthritis, endometriosis
10 and chronic inflammation. With respect to this use of the invention and especially with respect to the administration, the dosage and the manufacture of this pharmaceutical composition, the same applies as defined above.

For the context of these diseases, SEP inhibition aims at preventing the formation
15 of vascular vessels which support the diseased tissue. This, in turn, will reduce the amount of diseased or malignant cells (e.g. cancer cells). Accordingly, in a preferred embodiment of this use of the present invention, the inhibitor prevents the formation of vascular vessels in the tumor tissue.

20 The analysis in colon of SEP expression and VEGF expression, as shown in Figure 11, shows that expression of VEGF is found in tissues which have high expression of SEP. Vice versa, samples which have low expression of SEP, and therefore low activity of SEP, have low expression of VEGF. This may be a further hint that SEP regulates the expression of VEGF. Consequently, therapeutic
25 means that are capable of reducing the expression and/or activity of SEP can in turn cause reduced expression and hence reduced activity of VEGF.

Furthermore, as already discussed above and in Example 8, sSEP, SEP or the functional active derivative thereof are able to induce the production of VEGF.

- 26 -

Therefore, the inhibitor of the invention may act through the inhibition of the production of VEGF. Therefore, in a preferred embodiment of this use of the present invention, the inhibitor inhibits the production of VEGF.

- 5 In a further preferred embodiment, the inhibitor inhibits the production of IL-8 and/or RANTES.

As it is shown in Example 13, SEP expression is upregulated under hypoxic conditions. It is known in the art that during the growth of solid tumors, often hypoxic
10 conditions are found, which in turn result in the induction of new vascular vessels. SEP may be an important factor in this physiological process. In turn, inhibition of SEP function may result in maintaining the hypoxic conditions in the tumor, resulting in a suppression of tumor growth or even in a regression of tumor size.

- 15 Therefore, in a preferred embodiment of the use of the invention, the inhibitor prevents the formation of vascular vessels in the tumor tissue.

According to a preferred embodiment, the cancer is selected from the group consisting of brain cancer, pancreas carcinoma, stomach cancer, colon carcinoma,
20 skin cancer, especially melanoma, bone cancer, kidney carcinoma, liver cancer, lung carcinoma, ovary cancer, mamma carcinoma, uterus carcinoma, prostate cancer and testis carcinoma.

- The invention further includes a method for the treatment of a patient in need of
25 such treatment, wherein an effective amount of an inhibitor of SEP, sSEP or of a functional active derivative thereof is administered to the patient.

With respect to the preparation of this pharmaceutical composition, its administration and other embodiments the same apply as defined above.

- 27 -

Preferably, the inhibitor is used in combination with a VEGF inhibitor. In this case, the definition of an inhibitor is as mentioned above, only in the context of VEGF and not SEP.

- 5 The invention further relates to a method for the identification of a SEP inhibitor, wherein a potential inhibitor is tested for its activity to block the effects of SEP, sSEP or of a functional derivative thereof.

10 In this method of the invention, in general, SEP, sSEP or the corresponding gene are provided e.g. in an assay system and brought directly or indirectly into contact with a test compound, in particular a biochemical or chemical test compound. Then, the influence of the test compound on SEP, sSEP or the corresponding gene is measured or detected by measuring whether the SEP phenotype is reversed by addition of the potential inhibitor. Thereafter, suitable inhibitors can be analyzed
15 and/or isolated. For the screening of compound libraries, the use of High-Through-Put assays is preferred which are known to the skilled person or which are commercially available.

Suitable assays may be based on the gene expression of SEP or sSEP or on the
20 physiological activity of SEP or sSEP, i.e. the angiogenic properties.

For example, the following assay may be used for the identification of an inhibitor of the invention:

- 25
- transfection of SEP, mSEP (murine SEP; Xantos clone collection), hSEP (human SEP; received by RZPD clone services) into HEK293 cells
 - transfer of supernatants of HEK 293 cells onto HUVEC cells (as described for the screen in Example 1)
 - addition / incubation of HUVEC cells with LMW (low molecular weight)
30 compound library or other potential inhibitors
 - screening for inhibition of proliferating activity (reversion of phenotype)

- 28 -

- definition of lead structures
- analysis of specificity: inhibition of SEP, no direct effect on VEGF

The experimental steps transfection of HEK 293 cells, transfer of supernatant onto
5 HUVEC cells and screening for proliferation or inhibition of proliferation, respectively, can be carried out according to Examples 1 and 2.

The invention further relates to a method for the preparation of a pharmaceutical
composition, wherein an SEP inhibitor is identified as indicated above, synthesized in adequate amounts and finally formulated into a pharmaceutical composition.
10

Furthermore, the invention relates to the use of SEP, sSEP or a derivative thereof for the identification of proteins, that bind or interact with SEP, e.g. receptors or
15 pathway components, wherein

- a) a potential SEP interactor is brought into contact with SEP or a functional derivative thereof and
- b) binding of the potential interactor to SEP or the functional derivative thereof is determined.
20

An example for different strategies for providing an interactor of SEP is given in Example 6.

25 The following Figures and Examples are intended to illustrate further the invention without limiting it.

Short Description of the Figures:**Figure 1: Proliferation of HUVEC following transfer of supernatants from transfected 293 cells**

5

The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above. Vector represents the negative control resulting from transfection of the cloning vector pCMV-Sport6 into 293 cells and measurement of Alamar Blue to determine background proliferative effect of the supernatant derived from 293 cells. VEGF and PDGF were derived from the same clone collection to ensure compatibility of expression systems.

Figure 2: Proliferation of NHDF (normal human dermal fibroblasts) following transfer of supernatants from transfected HEK 293 cells

The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above. Vector represents the negative control resulting from transfection of the cloning vector pCMV-Sport6 into HEK 293 cells and measurement of Alamar Blue to determine background proliferative effect of the supernatant derived from 293 cells. VEGF and PDGF were derived from the same clone collection to ensure compatibility of expression systems. The results shown in Figs. 1 and 2 demonstrate that SEP acts specifically on endothelial but not on fibroblast cells.

- 30 -

Figure 3: Increased expression of SEP in tumor vs normal tissue and comparison to VEGF of tumor vs normal specificity

Database analyses reveals the frequencies of EST, 'hits' in public databases (NCBI CGAP, 5-16-03), which are indicative for relative expression levels in various normal and malignant tissues. Shown are normalized 'hit' frequencies per 200.000 EST entries x library. Note the different expression pattern in normal tissue (VEGF predominantly in uterus, SEP in placenta) and the decreased frequency and intensity of SEP hits in normal tissues.

Figure 4: Schematic domain structure of hSEP

Figure 4 shows the putative composition of the domains of hSEP. A globular domain containing Cysteins at the N-terminus is followed by a Prolin rich domain and two cleavage sites (arrows) for serum proteases / serin proteases, e.g. Thrombin, Plasmin or Urokinase. Repetitive units of similar Prolin containing sequences are followed by a Prolin rich domain and a trans-membrane domain.

Figure 5: Preferred soluble SEP fragments

This Figure shows preferred soluble SEP fragments of the invention.

Figure 6: Expression of human SEP in tumors vs. normal tissue by quantitative RT-PCR

Total RNA from mammary gland, and colon tissue was transcribed into cDNA and relative expression of SEP versus 18SrRNA was calculated after quantitative real-time PCR. Absolute expression levels have been analyzed by quantitative

- 31 -

real-time PCR for a panel of cDNAs from mammary gland and ovary tissue. Overexpression of SEP was observed in mammary and ovary cancer compared to normal tissue.

5

Figure 7: Production of VEGF in HEK 293 cells transfected with SEP

Figure 7 describes that HEK 293 cells transfected with SEP produce VEGF.

10

Figure 8: Proliferation of HUVEC following transfer of supernatants from transfected 293 cells

The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above. Vector represents the negative control resulting from transfection of the cloning vector pCMV-Sport6 into 293 cells and measurement of Alamar Blue to determine background proliferative effect of the supernatant derived from 293 cells. VEGF was derived from the same clone collection to ensure compatibility of expression systems. Expression of fragment 1-510 (1-510) showed the same activity compared to full length SEP (hSEP). There was also no difference in activity of SEP and the fragment 1-510 when tagged with the HA (hemagglutinin) epitope (hSEP HA; 1-510 HA).

25

Figure 9: Expression of human SEP in tumor vs normal tissue by quantitative RT-PCR

Total RNA from colon, lung, prostate and breast tissue was transcribed into cDNA and relative expression of SEP versus 18SrRNA was calculated after quan-

30

- 32 -

titative real-time PCR. Over-expression of SEP was observed in most colon, lung, prostate and breast cancer compared to normal tissue.

5 Figure 10: Expression of SEP and VEGF in breast tumors versus normal tissue
 by quantitative RT-PCR

Total RNA from breast tissue (cancer and normal) was transcribed into cDNA and relative expression of VEGF and SEP versus 18SrRNA was calculated after quantitative real-time PCR. Over-expression of SEP was observed more frequently in
10 breast cancer versus normal tissue compared to VEGF.

15 Figure 11: Increased expression of SEP in colon cancer versus normal tissues
 compared to VEGF

Total RNA from colon tissue (cancer and normal) was transcribed into cDNA and relative expression of SEP and VEGF versus G6PDH was calculated after quantitative real-time PCR. We observed a correlation between SEP and VEGF expression in normal colon tissue. In colon cancer the tissue where correlation is also
20 found, albeit less pronounced. Expression levels of SEP and VEGF correlated in normal colon tissue and less pronounced in colon cancer tissue.

25 Figure 12: Expression of h SEP in relation to G6PDH under hypoxic conditions
 by quantitative RT-PCR

Total RNA from HEK 293 cells either untreated (unt.) or incubated with medium containing 50mM CoCl₂ (CoCl₂) for 24 hours was transcribed into cDNA and
30 relative expression of SEP versus G6PDH was calculated after quantitative real-

- 33 -

time PCR. Induction of over-expression of SEP was observed in HEK 293 cells under hypoxic conditions.

5 Fig. 13: Expression of SEP in colon cancer vs. normal colon tissue

This figure shows an example of increased SEP expression in cancer tissue IHC of colon tissue samples. Immunoreactive cells are the malignant tumor cells. Staining for SEP protein was positive in the colon cancer tissue sample compared
10 to normal tissue where staining was negative.

Fig. 14: Expression of SEP on cell surface of transfected HEK293

15 HEK 293 transfected with hSEP showed specific staining for SEP protein on the cell surface in FACS analysis compared to control transfections with empty vector. The expression of SEP 1-510 on the cell surface is lower because the protein fragment is secreted.

20

Fig. 15 a: Proliferation of HUVEC following transfer of supernatants from transfected 293 cells

The relative fluorescence units (RFU) are given as mean value of three independent experiments. Experiments were performed following the manually adapted
25 protocol described above. Fragment 1-167 represents the negative control resulting from transfection of the expression plasmid into 293 cells and measurement of Alamar Blue to determine the non-specific (background) proliferative effect of the supernatant derived from HEK293 cells. The fragment 1-510 showed a similar
30 activity compared to full length SEP (SEP-Full). The fragment 1-167 showed no activity.

Fig. 15 b: Western Blot analysis for SEP of supernatants from transfected HEK293 cells

5

SEP protein in supernatant of transfected HEK293 was detected in Western Blot analysis using SEP specific antiserum. Lanes: 1=molecular weight standard, 2=SEP, 3=fragment 1-167, 4=fragment 1-236, 5=fragment 1-510. The indicated fragments were secreted into the supernatant of transfected HEK293 cells and
10 could be detected by SEP specific Western Blot Analysis.

Figure 15 c: Proliferation of HUVEC following addition of purified protein (eluates from nickel-agarose column)

15

Fragment 1-167 represents the negative control resulting from expression and purification of the inactive fragment from supernatants of transfected HEK293 cells. 1-167 was derived using the same expression and purification system. PBS represents a negative control to determine the non-specific (background) proliferative effect of the buffer the purified protein was dialysed against. The purified
20 fragment 1-510 showed activity compared to the negative controls. The fragment 1-167 showed no activity. The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above.

25

Figure 15 d: Western Blot analysis for SEP of purified protein

Purified SEP protein (eluates from nickel-agarose column) was detected in Western Blot analysis using SEP specific anti-serum. Lanes: 1=molecular weight stan-
30

- 35 -

dard, 2=fragment 1-167, 3=fragment 1-510. The indicated His tagged fragments were purified by metal chelate chromatography using nickel agarose.

5 Figure 16 a: Induction of IL-8 by SEP

Total RNA from HEK293 cells transfected with SEP or vector control was transcribed into cDNA and relative expression of IL-8 versus G6PDH was calculated after quantitative real-time PCR. Indicated is the relative induction of IL-8 by SEP compared to empty vector. Induction of IL-8 was observed by overexpression of SEP in HEK293 cells.

15 Figure 16 b: Induction of Rantes by SEP

Total RNA from HEK293 cells transfected with SEP or vector control was transcribed into cDNA and relative expression of Rantes versus G6PDH was calculated after quantitative real-time PCR. Indicated is the relative induction of Rantes by SEP compared to empty vector. Induction of Rantes was observed by overexpression of SEP in HEK293 cells.

20 Figure 16 c: Activity of purified supernatants from SEP transfected HEK293 cells

25

The relative fluorescence units (RFU) are given as mean value of three independent experiments. Experiments were performed following the manually adapted protocol described above. Supernatants of transfected HEK293 were separated on an Ion exchange column and the fractions were tested for their ability to induce proliferation/survival of HUVEC-cells. To see differences between supernatants of SEP-transfected and control vector transfected cells, AlamarBlue absorption

- 36 -

values of cells incubated with a certain fraction of the SEP-supernatants were divided by the corresponding value of cells incubated with fractions of control vector supernatants.

- 5 3 activity peaks can be found. The 3 peaks potentially reflect that not only SEP, but also additional factors like IL-8 and Rantes have proliferative activity.

Figure 17: Detection of SEP in ELISA with anti-SEP antibodies

10

An example of protein detection of soluble SEP by a rabbit anti-serum specific against SEP is shown. Supernatants of HEK293 cells transfected with indicated constructs were analysed as described above.

15

Figure 18: Inhibition of SEP induced HUVEC proliferation by anti-SEP anti-serum

- 20 Figure 18 shows an example of growth inhibition by a rabbit anti-serum specific against SEP. HUVEC cells were incubated with indicated amounts of anti-serum and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay (as described). Anti-SEP anti-serum reduced SEP induced growth of HUVEC cells compared to controls and is therefore suitable for therapeutic intervention of SEP activity.

25

Fig. 19: HUVEC growth inhibition by F(ab) 15

- 30 Figure 19 shows an example of growth inhibition by a human F(ab) specific against SEP generated by in vitro selection. HUVEC cells were incubated with 1

- 37 -

μg/ml of F(ab) and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay.

5 F(ab) 15 reduced SEP induced growth of HUVEC cells to 71% compared to untreated cells. Antibodies against SEP are therefore suitable for therapeutic intervention of SEP activity.

Figure 20: Inhibition of SEP induced HUVEC proliferation by SEP peptide

10

An example of growth inhibition by synthetic peptides derived from the extra cellular domain of SEP is shown. HUVEC cells were incubated with indicated amounts of peptide and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay (as described). P3061 and pWobble reduced SEP induced proliferation of HUVEC cells compared to untreated cells. Therefore peptides derived from SEP are suitable for therapeutic intervention of SEP activity.

15

Figure 21 a: Induction of HUVEC proliferation by stable MCF-7 clones

20

The relative fluorescence units (RFU) are given as mean value of three independent experiments. Experiments were performed following the manually adapted protocol described above. Stably SEP overexpressing clone MCF-7 SEP40 showed induction of proliferative activity on HUVEC compared to a stable control clone MCF-7 Vector 20.

25

Figure 21 b: Induction of IL-8 by stable MCF-7 clones

30 Total RNA from MCF-7 cells stably transfected with SEP or vector control was transcribed into cDNA and relative expression of IL-8 versus G6PDH was calcu-

- 38 -

lated after quantitative real-time PCR. Indicated is the relative induction of IL-8. Stably SEP overexpressing clone MCF-7 SEP40 showed increased induction of IL-8 compared to a stable control clone MCF-7 Vector 20.

5

Figure 21 c: Induction of Rantes by stable MCF-7 clones

Total RNA from MCF-7 cells stably transfected with SEP or vector control was transcribed into cDNA and relative expression of Rantes versus G6PDH was calculated after quantitative real-time PCR. Indicated is the relative induction of Rantes. Stably SEP overexpressing clone MCF-7 SEP40 showed increased induction of IL-8 compared to a stable control clone MCF-7 Vector 20.

15 Figure 21 d: Induction of HUVEC proliferation by stable PC-3 clones

The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above. The clone PC-3 510-1 stably overexpressing the fragment 1-510 showed induction of proliferative activity on HUVEC compared to a stable control clone PC-3 Vector 7.

Figure 21 e: Induction of IL-8 by stable PC-3 clones

25

Total RNA from PC-3 cells stably transfected with fragment 1-510 or vector control was transcribed into cDNA and relative expression of IL-8 versus G6PDH was calculated after quantitative real-time PCR. Indicated is the relative induction of IL-8. The clone PC-3 510-1 stably overexpressing the fragment 1-510 showed increased induction of IL-8 compared to a stable control clone PC-3 Vector 7.

30

Figure 21f: Induction of Rantes by stable PC-3 clones

Total RNA from PC-3 cells stably transfected with fragment 1-510 or vector control was transcribed into cDNA and relative expression of Rantes versus G6PDH
5 was calculated after quantitative real-time PCR. Indicated is the relative induction of Rantes. The clone PC-3 510-1 stably overexpressing the fragment 1-510 showed increased induction of Rantes compared to a stable control clone PC-3 Vector 7.

10

EXAMPLES

15 **Example 1: Isolation of the SEP cDNA by expression screening**

An expression screen was conducted in order to isolate novel cDNAs that encode secreted proteins which stimulate endothelial cell proliferation. Plasmid DNAs were prepared on Xantos' proprietary high-throughput robot assembly according
20 to standard Xantos protocols (see WO 03/014346):

Bacteria in growth plates were sedimented by centrifugation and supernatant was exhausted. The pellets were then resuspended with RNase containing buffer (P1), an alkaline buffer (P2) was added for lysis and afterwards neutralized by an acid
25 buffer (P3).

After a short incubation, plates were again centrifuged and the supernatant transferred into additional plates. To clear the suspension from bacterial endotoxins buffer P4 was added and mixed. The supernatants of an additional centrifugation
30 were then transferred to third plate and mixed with silica to bind plasmid DNA. The silica was washed, for this, the plate was centrifuged and the pellets were

- 40 -

resuspended with acetone on a plate shaker. The plates were again centrifuged and the acetone was exhausted and evaporated. The DNA was eluted by mixing the dry silica pellet with water (60°C) and after a centrifugation step the DNA containing supernatant was transferred into the last plate.

5

For incubation and mixing a plate shaker was used and the buffers were added using an eight channel dispenser.

(P1: Tris EDTA with RNase, P2: NaOH / SDS, P3: potassium acetate, P4: SDS
10 in isopropanol)

To facilitate the production of the proteins encoded by individual cDNA clones, 2.2×10^4 HEK 293 cells were seeded in 96-well tissue culture plates (Costar) in 100µl DMEM medium containing 5% FCS (Invitrogen). Transfection of 18000
15 cDNAs from a clone collection (MGC Clone Collection (IRAK-Collection („Mammalian Gene Collection“; RZPD, Berlin) described in Strausberg RL, Feingold EA, Klausner RD, Collins FS. The Mammalian Gene Collection. Science, 1999, 286, 455-457) on 293 cells was performed 24hrs post seeding using calcium phosphate co-precipitation. Precipitates were removed after 4 hours
20 and cells were switched to nutrient deficient DMEM (DMEM, 1.5%FCS, 1% Na-pyruvate, 1% Glutamine, 100µg/ml gentamycin, 0.5µg/ml amphotericin B). Human umbilical cord vein endothelial cells (HUVEC) were cultured in ECGM with supplements (Promocell Heidelberg, single quotes) containing 1 % serum, 50µg/ml gentamycin, 0.4µg/ml amphotericin B and 50U/ml nystatin. HUVECS were plated
25 at 2.5×10^3 cells /well on day 3. Before transfer of supernatants on day 4, 90µl of medium was removed, HUVECS were washed once with 200µl of PBS, then 75µl of nutrient deficient medium (ECBM, with supplements, Promocell, Heidelberg) containing 1µg/ml hydrocortisol, 50µg/ml gentamycin, 0.4µg/ml amphotericin B and 50U/ml nystatin was added following 25µl of supernatants from the trans-
30 fected 293 cells. Supernatants were incubated for 4 days on HUVEC cells. Read-out was performed using Alamar Blue (Biosource, California USA). For each well

- 41 -

of a 96well plate, 11 μ l of Alamar Blue reagent were mixed with 9 μ l of ECBM and the resulting 20 μ l were added directly to the HUVEC cells without removal of medium. Incubation was performed at 37°C for 4 hours. Alamar Blue fluorescence was measured at 530nm excitation and 590nm emission.

5

Positive control for proliferation of HUVECs was supernatant containing VEGF derived from the clone collection. Negative controls were supernatants from vector-transfected cells and PDGF-transfected 293 cells.

- 10 This screen led to the isolation of a cDNA which will be referred to as Stimulator of Endothelial Proliferation, SEP. The original SEP clone identified was the IMAGE clone 5123637 derived from a murine liver cDNA library. To identify a human orthologue for mSEP, BLAST searches against the human UniGene data-
- 15 base were performed. They revealed the presence of the mRNA sequence of the hypothetical protein KIAA1271 with a low E-value of about 1e-25. On amino acid level, however, the E-value increases to 5e-125 with an overall homology of 50% between the murine and the human predicted proteins. The assumption that the respective genes may be orthologous is supported by chromosomal localisation studies: the mouse locus of 5123637 is syntenic to the human locus of
- 20 KIAA1271, 2F2, and 20p13 respectively.

Example 2: Verification of proliferation-inducing activity

- 25 For the verification of the proliferation-inducing activity of SEP, mSEP (murine SEP; Xantos clone collection), hSEP (human SEP; received by RZPD clone services) and controls were transfected into HEK293 cells and supernatants were transferred onto HUVEC as described for the screen (Example 1) except that all manipulations were carried out manually. Figure 1 shows the proliferation-
- 30 inducing activity of mSEP and hSEP in comparison to VEGF.

- 42 -

Example 3: Verification of specific expression

In order to investigate the cell type specificity of SEP supernatants from trans-
5 fected HEK 293 cells, they were also added to normal human dermal fibroblasts
(NHDF). NHDF were seeded at 1,000 cells per well on 96-well tissue culture
plates two days prior to the transfer in 100µl complete Fibroblast Growth Medium
(Promocell, Heidelberg). 24h prior to the transfer the medium was changed to
100µl Fibroblast Basal Medium (Promocell, Heidelberg) containing 75µg/ml gen-
10 tamycin, 50ng/ml amphotericin B. After 25µl of 293 HEK supernatant had been
transferred cells were incubated for 4 days and viable cell number was assessed by
Alamar Blue reduction as above. Figure 2 demonstrates that mSEP and hSEP
were unable to stimulate NHDF proliferation to levels above empty vector con-
trols. However, the cells were clearly responsive to supernatants containing FGF-
15 2 or PDGF. These results demonstrate that SEP acts specifically on endothelial
but not fibroblast cells.

Example 4: Expression analysis of hSEP in comparison to VEGF

20 Expression analyses of human SEP and VEGF were performed using the Ex-
pressed Sequence Tag data provided by the Cancer Genome Anatomy Project of
the National Cancer Institute, Bethesda, Maryland, USA. SEP was represented by
Unigene Cluster Hs.183669 and VEGF was represented by Hs.73793 of Unigene
25 build Hs.160. EST frequencies per tissue were normalized to 200,000 total EST
per tissue. Pooled tissues and tissues for which both the VEGF and SEP fre-
quency were zero were excluded from the analyses. The results are shown in Fig-
ure 3.

30

Example 5: Structure and separate functional domains of SEP

The primary amino acid sequence of SEP (seqID AAH44952.1) forms a protein of 540 amino acids (estimated size 59.4 of kDa), which is anchored to the membrane by a carboxyterminal membrane spanning domain followed by a hydrophilic stop-transfer sequence at the C-terminal end of the molecule. Further details related to the domain structure of SEP are provided in Figure 4. Extracellular domains, which appear to be separated from each other by flexible Gly/Ser rich interdomain linker sequences include repeats which contain 4x multiples of the sequence (L/V)-P-S-K-(LV)-P-T, as well as additional proline rich modules. The amino terminal domain contains multiple cysteins which can form disulfide bonds. Of particular interest is the observation that two very flexible and hence exposed sequence stretches at position 180-2 and 255-8 are preceded by arginine rich sequences at position 165-72 and 231-40. Although these sequences are not identified as specific 'classical' consensus sequences for recognition by extracellular or serum proteases per se, they can be considered to provide exposed sensitive sites for proteolytic processing of SEP. A further protease sensitive site may be located directly preceding the C-terminal transmembrane domain at position 509-14. Examples of products of proteolytic processing of SEP by surface-bound or extracellular proteases are represented by sequence ID's 7 to 17.

It has to be noted that the N-terminal protein fragments of SEP, as well as all those that have become separated from the transmembrane domain from the extracellular side, are to be considered as soluble extracellular proteins and peptides. These products can express their biological function at the site of production (highest extracellular concentration) as well as at nearby and remote locations which are different from their side of production.

Example 6: Identification of SEP interacting protein**A) General strategy for the identification of SEP interacting protein****5 Step 1:**

Perform database search and find published interactor. Confirm published interactor by selective knock-out (RNAi) in that cellular assay SEP was defined in.

Step 2:

10 Prerequisite: Get an antibody against SEP or fuse SEP with another protein/peptide that could be either a reporter gene (e.g. GFP or enzyme or radioactive label or other chemical compound) or immunoprecipitable by an antibody. The fusions could be checked for maintained binding properties in the original functional assay.

15

a) A second transfection screen: prepare a cDNA library from a transcriptome compromising the interactor (e.g. the transcriptome of the cell SEP was found functional on). Transfect and over express the cDNA clones individually into an interactor-negative cellular background (this could be checked in advance with the fusion-constructs). Detect labeled cells by visual, enzymatic or physical methods targeted to the fusion-partner of SEP. Gain interactor cDNA from cDNA stock.

20

b) A co-precipitation approach followed by mass spectrometric analysis of bound partners. Optional: Confirm cellular localisation with labeled ligand. Extract the whole cellular extract or the appropriate cellular compartment by precipitating the interactor with SEP. Precipitation could be performed by

25

- 45 -

immobilization via SEP specific antibodies or immobilization of SEP via a fused protein, peptide or chemical label.

[Precipitation of membrane proteins might demand

- 5 - Special solubilisation conditions (e.g. detergent concentrations) that have to be changed prior to addition of SEP and immobilization-compound.
- Cross-linking of SEP and interactor to preserve the interaction.]

The precipitate could be processed in the following ways:

- 10 i) Separation on protein gels and blotting (optional: proteolytic cleavage prior to or after electrophoresis). Subsequently mass-spectrometric analysis is performed followed by comparison of peptide data with appropriate mass-spec-databases. In case of no such peptide-map-database entry: sequencing of protein spot or cleavage derived peptides and search in protein and nucleic acid databases (with derived nucleic acid sequences according to the translation code; e.g. search in EST-databases).
- 15 ii) Immunisation of animals with precipitated complex or derived parts of it in order to get antibodies against the putative interactor. These antibodies could serve in reverse immuno-precipitations as tools to show interaction between the respective antigen and SEP.
- 20

Step 3:

Perform in vivo and in vitro protein-protein binding studies:

- 25 a) Yeast or mammalian two-hybrid assay with SEP as bait and a cDNA library cloned into the corresponding pray-vector. The pray-cDNA library should be derived from cells showing SEP exerted function.

- 46 -

- b) Phage display hybridization with recombinant and labeled SEP
- c) Hybridization of protein chips with recombinant and labeled SEP

5 Step 4:

- a) A second transfection screen: prepare a cDNA library from a transcriptome compromising the interactor (e.g. the transcriptome of the cell SEP was found functional on). Transfect and over express the cDNA clones individually into a cellular background negative for interactor expression and SEP
10 function (this could be checked in advance with the fusion-constructs and antibodies). Detect SEP function / activation in these cells by monitoring SEP induced phenotype (e.g. induction of VEGF). Gain interactor cDNA from cDNA stock.
- b) A supernatant screen: prepare a cDNA library from a transcriptome compromising the interactor (e.g. the transcriptome of the cell SEP was found
15 functional on). Transfect and over express the cDNA clones individually into a cellular background potentially negative for interactor expression. Transfer supernatant (containing secreted protein coded by the transfected
20 cDNA) to cells positive for SEP expression. Detect SEP function / activation in these cells by monitoring SEP induced phenotype (e.g. induction of VEGF). Gain interactor cDNA from cDNA stock.

25 B) Variants of identification of SEP interacting proteins depending on the properties of SEP:

1. Identification of a ligand type SEP interactor

In this variant the following steps could be performed in parallel or alternatively:

- 47 -

Step 1, step 2b, step 3a+b+c, step 4a+b

2. Identification of a co-receptor type SEP interactor

Step 1, step 2b, step 3a+b+c, step 4a

5

3. Identification of a receptor type SEP interactor

Step 1, step 2a+b, step 3a+b+c, step 4a

10 **Example 7: Increased expression of SEP in mammary and ovary cancer compared to normal tissue**

Figure 3 indicates that EST data show high expression of human SEP in cancer versus normal in most tissues. For this, expression levels of SEP in RNAs and
15 cDNAs from human mammary gland (normal and cancer), ovary (normal and cancer) and colon (normal and cancer) were analysed by quantitative real-time PCR. cDNA was synthesized from 1 µg of total RNA in a volume of 20 µl using random hexamers as primer and AMV Reverse Transcriptase (Roche Diagnostics). Real-time PCR was carried out using a LightCycler (Roche Diagnostics). Reac-
20 tions were set up in microcapillary tubes using the following final concentrations: 1 µM each of SEP sense (TCA GGA GCA GGA CAC AGA AC) and SEP antisense (TGG AAG GAG ACA GAT GGA GAC) primers, 3 µM MgCl₂, 1x SYBR Greenmaster mix and 0,2 µl of cDNA. Cycling conditions were as follows: denaturation (95° C for 10 min), amplification and quantitation (95°C for 10 s,
25 56°C for 10 s and 72°C for 13 s, with a single fluorescence measurement at the end of the 72°C for 13 s segment) repeated 45 times. A melting curve program (55–95°C with a heating rate of 0.1 ° C/s and continuous fluorescence measurement) and a cooling step to 40°C followed. For relative quantification the proce-

- 48 -

ture was repeated for 18S rRNA as reference gene. Data were analyzed using LightCycler analysis software.

In complete agreement with the computer prediction shown in figure 3 we observed higher expression of SEP in mammary and ovarian cancer compared to normal tissue. Also, in agreement with figure 3, colon samples showed a high expression of SEP in tumor as well as in normal tissue. (see figure 6)

10 **Example 8: Induction of VEGF**

Induction of VEGF by SEP was measured in an ELISA specific for detection of hVEGF. 2×10^4 HEK 293 cells were transfected in parallel with 0.28 μ g of the indicated cDNAs (see Fig. 7) and grown in serum reduced culture medium (1.5% FCS). Concentration of hVEGF in the supernatant was determined 48h after transfection according to the manufacturers protocol (PromoKine - Human VEGF ELISA Kit, PromoCell GmbH, Heidelberg, Germany). The empty vector pCMVSPORT6 was used as negative control. As positive control cells were transfected with an expression plasmid for hVEGF. Shown are means of 4 independent experiments.

The induction of hVEGF by SEP and/or its murine orthologue is significantly higher compared to the vector control (8 to 13 fold). The concentration of hVEGF in supernatants of SEP transfected cells is similar to cells transfected with the expression plasmid for hVEGF.

Example 9: Verification of proliferation-inducing activity of fragment 1-510

For the verification of the proliferation-inducing activity of fragment 1-510 of SEP (expression plasmid for this fragment), SEP (human SEP) and controls were transfected into HEK293 cells and supernatants were transferred onto HUVEC as described for the screen (Example 1) except that all manipulations were carried out manually. Figure 8 shows the proliferation-inducing activity of SEP and fragment 1-510 in comparison to VEGF. The fragment has the same degree of activity as the full length SEP.

10

Example 10: Increased expression of SEP in colon, lung, prostate and breast cancer compared to normal tissue

Figure 9 indicates higher expression of human SEP in cancer versus normal tissues. For this, expression levels of SEP in RNAs and cDNAs from human colon (normal and cancer), lung (normal and cancer), prostate (normal and cancer) and breast (normal and cancer) were analysed by quantitative real-time PCR. cDNA was synthesized from 1 µg of total RNA in a volume of 20 µl using random hexamers as primer and AMV Reverse Transcriptase (Roche Diagnostics). Real-time PCR was carried out using a LightCycler (Roche Diagnostics). Reactions were set up in microcapillary tubes using the following final concentrations: 1 µM each of SEP sense (TCA GGA GCA GGA CAC AGA AC) and SEP antisense (TGG AAG GAG ACA GAT GGA GAC) primers, 3 µM MgCl₂, 1x SYBR Greenmaster mix and 0,2 µl of cDNA. Cycling conditions were as follows: denaturation (95° C for 10 min), amplification and quantitation (95°C for 10 s, 56°C for 10 s and 72°C for 13 s, with a single fluorescence measurement at the end of the 72°C for 13 s segment) repeated 45 times. A melting curve program (55–95°C with a heating rate of 0.1 ° C/s and continuous fluorescence measurement) and a cooling step to 40°C followed. For relative quantification the procedure was re-

- 50 -

peated for 18S rRNA as reference gene. Data were analyzed using LightCycler analysis software.

In agreement with the computer prediction shown in figure 3 we observed higher
5 expression of SEP in mammary, prostate and lung cancer compared to normal tissue. Furthermore, also for colon cancer a higher SEP expression was found.

**Example 11: Increased expression of SEP in breast cancer versus normal
10 tissues compared to VEGF**

Figure 10 indicates higher expression of human SEP in more breast cancer versus normal tissues compared to VEGF. For this, expression levels of VEGF in RNAs and cDNAs from human breast (normal and cancer) were analysed by quantitative
15 real-time PCR in the same breast tissue samples as indicated in figure 9. cDNA was synthesized from 1 µg of total RNA in a volume of 20 µl using random hexamers as primer and AMV Reverse Transcriptase (Roche Diagnostics). Real-time PCR was carried out using a LightCycler (Roche Diagnostics). Reactions were set up in microcapillary tubes using the following final concentrations: 1 µM
20 each of VEGF sense (TAC CTC CAC CAT GCC AAG TG) and VEGF antisense (CTA CTA AGA CGG GAG GAG GAA G) primers, 3 µM MgCl₂, 1x SYBR Greenmaster mix and 0,2 µl of cDNA. Cycling conditions were as follows: denaturation (95° C for 10 min), amplification and quantization (95°C for 10 s, 56°C for 10 s and 72°C for 13 s, with a single fluorescence measurement at the end of
25 the 72°C for 13 s segment) repeated 45 times. A melting curve program (55–95°C with a heating rate of 0.1 ° C/s and continuous fluorescence measurement) and a cooling step to 40°C followed. For relative quantification the procedure was repeated for 18S rRNA as reference gene. Data were analyzed using LightCycler

- 51 -

analysis software. Relative expression levels of VEGF were compared to relative expression levels of SEP as shown in figure 9.

We observed in more cases higher expression of SEP in breast cancer versus normal tissue compared to VEGF in breast cancer versus normal tissue.

Example 12: Increased expression of SEP in colon cancer versus normal tissues compared to VEGF.

10

Figure 11 indicates correlating expression levels of human SEP and VEGF in normal colon tissue where as correlation is less pronounced in colon cancer. For this, expression levels of VEGF in RNAs and cDNAs from human colon (normal and cancer) were analyzed by quantitative real-time PCR (as described in figures 9 and 10). Relative expression levels of SEP were compared to relative expression levels of VEGF as shown in Figure 9.

We observed a correlation between SEP and VEGF expression in normal colon tissue compared to colon cancer tissue where correlation is less pronounced.

20

Example 13: Increased expression of SEP in HEK 293 cells under hypoxic conditions.

Figure 12 indicates higher expression of human SEP in HEK293 cells under hypoxic conditions simulated by incubation with CoCl_2 compared to expression levels of G6PDH. For this, expression levels of SEP in RNAs and cDNAs from HEK

- 52 -

293 cells either untreated or incubated with medium containing 50mM CoCl₂ for 24 hours were analyzed by quantitative real-time PCR (as described in example 10). Incubation with CoCl₂ is an accepted model for chemical induction of hypoxic conditions in cells. In the same experiment expression levels of VEGF were determined under identical conditions. For relative quantification the procedure was repeated for G6PDH as reference gene. Data were analyzed using LightCycler analysis software.

The data of this experiment show that induction of over-expression of SEP was observed in HEK 293 cells under hypoxic conditions. The degree of induction was in the same range of hypoxic induction of VEGF expression.

Example 14: Increased expression of SEP in tumor tissue

For the analysis of SEP expression in tumor tissue, tissue samples of patients (normal and tumor tissue) were stained for SEP protein using immuno histochemistry (IHC) and mRNA levels were measured using quantitative real-time PCR (QPCR) as described in example 7. Table 1 shows expression of SEP in different solid tumors and corresponding normal tissues. Figure 13 shows increased expression of SEP in colon tumor tissue compared to adjacent normal tissue as an example.

Table 1: Expression of SEP in different normal and tumor tissue

Tissue	Normal (IHC)	Normal (QPCR)	Cancer (IHC)	Cancer (QPCR)
Breast	-	- (mammary gland)	+	+
Colon	-	-	+/++	+
Lung	-	-	+/-	+
Prostate	-	+/-	n.d.	+

n.d. not determined

- 53 -

Indicated tissue samples were either stained for SEP protein by immuno histo-chemistry using anti-SEP antiserum (described in Example 18) or were analysed for SEP RNA expression by QPCR as described in Example 7.

- 5 Immunostaining (Applied Phenomics, Estonia) was performed on whole body tissue arrays (core diameter 0.6 and 1.5 mm, paraformaldehyde-fixed and paraf-
fin-embedded material). Manual immunostaining using DAKO secondary re-
agents (DAKO Duet HRP kit) was performed using standard citrate /microwave
pre-treatment. Unspecific binding of secondary reagents was prevented by biotin
10 blocking. The results were evaluated by experts in immunohistochemistry and a
pathologist.

The immunoreactive cells were the tumor cells while the surrounding stroma was
essentially negative. In some instances, sporadic staining was detected in some
15 capillaries of the tumor tissue (not normal), which was in the endothelial cells or
smooth muscle cells (Fig. 13). Expression of SEP is increased in tumor tissue of
breast, colon, lung and prostate. Normal tissue tested by IHC was found positive
for pancreas and salivary gland and negative for brain, peripheral nerve, adrenal
gland, ovary, testis, thyroid, bone marrow, spleen, tonsils, myocard, aorta, vena
20 cava, liver, esophagus, stomach, small intestine, kidney, bladder, uterus, cervix,
skeletal muscle, skin, lymph node and adipose tissue.

Example 15: Expression of SEP on the cell surface of transfected HEK 293

25

To verify expression of SEP on the cell surface, HEK 293 cells were transfected
with expression plasmids of indicated constructs and expression of SEP was
monitored by FACS analysis using anti-SEP antiserum. Expression of SEP was

- 54 -

analysed by binding of anti-SEP antibodies (antiserum) to the cell surface of transfected HEK293 and detection by FACS. For this, HEK293 cells were transfected with SEP, fragment 1-510 or empty vector as control. 48 h after transfected cells were harvested, washed 3 times with PBS containing 0.1 % BSA (bovine serum albumine) and stained with anti-SEP antiserum (rabbit, 1:1000 in PBS/0.1%BSA, 1 h on ice). After washing 3 times with PBS/0.1%BSA the secondary antibody (Dianova, FITC labeled anti rabbit, 1:100) was applied for 30 min on ice. Before FACS analysis cells were incubated with propidium iodine (PI) for detection of dead cells. Cells were analysed in a FACSCalibur cytometer (Becton Dickinson) for binding of anti-SEP antiserum to living (PI) negative cells using FACS analysis software. To determine background staining of the secondary antibody cells were incubated without specific anti-SEP antibodies (primary antibody). Specific surface signal was calculated as % positive cells in the presence of primary antibody compared to % positive cells in the absence of primary antibody.

HEK 293 transfected with hSEP showed specific staining for SEP protein on the cell surface in FACS analysis compared to control transfections with empty vector. The expression of SEP 1-510 on the cell surface is lower because the protein fragment is secreted (Fig. 14)

Example 16: SEP is active as soluble/shedded protein

For the verification of the proliferation-inducing activity of fragment 1-510 of SEP, His-tagged fragments of SEP and controls were transfected into HEK 293 cells and protein was purified from the supernatants by metal chelate chromatography (nickel agarose) and transferred onto HUVEC. For this, HEK293 cells were transfected with CaCl₂ in with SEP 1-510 cDNA or 1-167 and full length as

- 55 -

controls. After transfection the medium was exchanged against DMEM containing 0.5 % FCS plus supplements. After 48 hours supernatants were collected and analysed for proliferative activity (Figure 15a) and protein (Figure 15b). For protein purification supernatants were centrifuged (5000 g, 10 min) and adjusted to final concentrations of 10 % glycerol and 150 mM NaCl. The protein solutions were bound to nickel agarose (1 ml, Pharmacia) for 2 h at 4 °C. After washing with PBS tagged proteins were eluted with 250 mM imidazole. Fractions were collected, dialysed against PBS and analysed for proliferative activity on HUVEC (Figure 15c) and protein (Figure 15d). Proliferation assays were performed as described. For Western Blot analysis proteins were separated on a SDS PAGE and transferred to a nitro cellulose membrane. SEP protein was detected by anti-SEP antiserum (rabbit, 1:2000) and alkaline phosphates conjugated anti rabbit antibody (Promega, 1:7500).

Figure 15a shows the proliferation-inducing activity of purified fragment 1-510 in comparison to PBS and the inactive fragment 1-167. The relative fluorescence units (RFU) are given as mean value from three independent experiments. Experiments were performed following the manually adapted protocol described above. Fragment 1-167 represents the negative control resulting from transfection of the expression plasmid into 293 cells and measurement of Alamar Blue to determine the non-specific (background) proliferative effect of the supernatant derived from HEK293 cells. The fragment 1-510 showed a similar activity compared to full length SEP (SEP-Full). The fragment 1-167 showed no activity.

For the verification of secretion of SEP, SEP was transfected in HEK 203 cells and the supernatant was analysed in Western blot analysis. SEP protein in supernatant of transfected HEK293 was detected in Western Blot analysis using SEP specific antiserum (Fig 15 b). The indicated fragments were secreted into the supernatant of transfected HEK293 cells and could be detected by SEP specific Western Blot Analysis.

- 56 -

To analyse the activity of the purified proteins, eluates from nickel-agarose column were applied to HUVEC cells and proliferation was monitored using Alamar Blue Assay. The relative fluorescence units (RFU) are given as mean value of three independent experiments. Experiments were performed following the manually adapted protocol described above (Figure 15c). Fragment 1-167 represents the negative control resulting from expression and purification of the inactive fragment from supernatants of transfected HEK293 cells. 1-167 was derived using the same expression and purification system. PBS represents a negative control to determine the non-specific (background) proliferative effect of the buffer the purified protein was dialysed against. The purified fragment 1-510 showed activity compared to the negative controls. The fragment 1-167 showed no activity.

To verify SEP protein nickel column eluates were analyzed in Western Blot analysis using SEP specific antiserum. Purified SEP protein (eluates from nickel-agarose column) was detected in Western Blot analysis using SEP specific antiserum (Figure 15d).

Example 17: SEP induces pro-angiogenic factors

Expression analysis of HEK 293 cells transfected with SEP in comparison to cells transfected with vector as control were performed to analyse whether additional pro-angiogenic factors are induced by SEP. For this, total RNA from transfected cells was analysed using Affymetrix Chip analysis. Besides several small inducible cytokines the known pro-angiogenic proteins IL-8 and Rantes were induced by SEP. To verify this observation expression of IL-8 and Rantes was analysed in HEK293 cells transfected with SEP using quantitative real-time PCR. Figures

- 57 -

16 a and 16 b indicate increased expression of IL-8 and Rantes in HEK293 cells transfected with SEP compared to vector control. For this, cDNA was synthesized from 1 µg of total RNA in a volume of 20 µl using random hexamers as primer and AMV Reverse Transcriptase (Roche Diagnostics). Real-time PCR was carried out using a LightCycler (Roche Diagnostics). For analysis of Rantes reactions were set up in microcapillary tubes using the following final concentrations: 1 µM each of Rantes sense (CGC TGT CAT CCT CAT TGC TA; SEQ ID NO: 19) and Rantes antisense (GCA CTT GCC ACT GGT GTA GA; SEQ ID NO: 20) primers, 2.5 µM MgCl₂, 1x SYBR Greenmaster mix and 0,2 µl of cDNA. Cycling conditions were as follows: denaturation (95° C for 10 min), amplification and quantitation (95°C for 10 s, 55°C for 10 s and 72°C for 13 s, with a single fluorescence measurement at the end of the 72°C for 13 s segment) repeated 45 times. A melting curve program (55–95°C with a heating rate of 0.1 ° C/s and continuous fluorescence measurement) and a cooling step to 4°C followed. For analysis of IL-8 reactions were set up in microcapillary tubes using the following final concentrations: 1 µM each of IL-8 sense (CTG CGC CAA CAC AGA AAT TA; SEQ ID NO: 21) and IL-8 antisense (TGA ATT CTC AGC CCT CTT CA; SEQ ID NO: 22) primers, 2.5 µM MgCl₂, 1x SYBR Greenmaster mix and 0,2 µl of cDNA. Cycling conditions were as follows: denaturation (95° C for 10 min), amplification and quantitation (95°C for 10 s, 58°C for 10 s and 72°C for 13 s, with a single fluorescence measurement at the end of the 72°C for 13 s segment) repeated 45 times. A melting curve program (55–95°C with a heating rate of 0.1 ° C/s and continuous fluorescence measurement) and a cooling step to 4°C followed. For relative quantification the procedure was repeated for G6PDH RNA as reference gene. Data were analyzed using LightCycler analysis software. Figure 16c shows multiple proliferative activities of supernatants of HEK293 cells transfected with SEP separated by ion exchange chromatography. For this, HEK293 cells were transfected with CaCl₂ in 10 cm plates with SEP full length cDNA or the empty vector as a control. After transfection the medium was exchanged against DMEM containing 0.5% FCS plus supplements. After 48 hours supernatants were collected, centrifuged (5000 g, 10 min) and 15 ml of supernatant was combined with

- 58 -

30 ml of anion exchange loading buffer (20 mM Tris pH8) (load). The protein solution was loaded onto a HiTrap Q FF column (1 ml, Pharmacia) using an ÄKTA-FPLC system (also Pharmacia). The flowthrough was collected and the protein was eluted by applying a 0 – 400 mM NaCl gradient. 46 fractions were collected and 10 ul of each fraction was applied to a 96 well of HUVEC cells (2,8x10³ cells per well, plated 24 hours earlier). A proliferation assay was performed as described above.

Figure 16 c shows that 3 activity peaks (starting at 190 mM, 260 mM and 350 mM NaCl) can be found if the supernatant from SEP-transfected cells is compared with the supernatant from control vector transfected cells.

Example 18: Generation of antibodies against SEP and detection of SEP protein in Western Blots

Antibodies against SEP were produced by immunizing rabbits with peptides PMPVQETQAPESPGENSEQAL (SEQ ID NO: 23) and PADPDGGPRPQADRK (SEQ ID NO: 24). Additionally, rats were immunized with the peptide SKLPINSTRAGM (Eurogentec, Belgium; SEQ ID NO: 25). In vitro selection of human F(ab)s was performed using recombinant SEP (aa 37-510) from E.coli using phage display according to Kretzmar and von Ruden (Curr Opin Biotechnol. 2002 Dec;13(6):598-602). The antibodies were tested in Western Blot and ELISA to show suitability for diagnostic assays. Proliferation assays with HUVEC cells were performed to determine functional activity of the antibodies (growth inhibition).

- 59 -

Table 2: Antibodies against SEP

Antibody	Type	Detects SEP in Western Blot	Detects SEP in ELISA	Inhibition of HUVEC growth
1	Human F(ab)	+	+	-
2	Human F(ab)	+	+	+
3	Human F(ab)	+	+	-
4	Human F(ab)	+	+	+
5	Human F(ab)	+	+	-
6	Human F(ab)	+	+	-
7	Human F(ab)	+	+	-
8	Human F(ab)	+	+	-
9	Human F(ab)	+	+	-
10	Human F(ab)	+	+	-
11	Human F(ab)	+	+	-
12	Human F(ab)	+	+	-
13	Human F(ab)	+	+	-
14	Human F(ab)	+	+	-
15	Human F(ab)	+	+	+
Rabbit serum	Polyclonal serum	+	+	+
Affinity puri- fied rabbit serum 1	Polyclonal serum	+	-	+
Affinity puri- fied rabbit serum 2	Polyclonal serum	+	-	+
Rat serum 1	Polyclonal serum	+	+	n.d.
Rat serum 2	Polyclonal serum	+	+	n.d.
Rat serum 3	Polyclonal serum	+/-	+	n.d.

Antibodies against SEP recognise SEP protein and are suitable for diagnostic detection of SEP.

- 60 -

Example 19: Detection of SEP in ELISA

Antibodies against SEP were produced by immunizing rabbits with peptides PMPVQETQAPESPGENSEQAL (SEQ ID NO: 23) and PADPDGGPRPQADRK
5 (SEQ ID NO: 24). Additionally, rats were immunized with the peptide SKLPINSTRAGM (SEQ ID NO: 25; Eurogentec, Belgium). In vitro selection of human F(ab)s was performed using recombinant SEP (aa 37-510) from E.coli. The antibodies were tested in ELISA to show suitability for diagnostic assays. For this, HEK293 cells were transfected with an expression plasmid for SEP 1-510
10 tagged with the V5-epitope or with empty vector as control. 48 h after transfection supernatants were collected and secreted protein was bound to 96 well plates via the anti-V5 antibody (Invitrogen). Bound SEP protein was detected using anti-SEP antiserum (1:1000) and peroxidase conjugated antirabbit antibody (Dianova, 1:5000). Incubation with pre-immunserum from the same rabbit served as nega-
15 tive control. Figure 17 shows an example of protein detection of soluble SEP by a rabbit anti-serum specific against SEP. Supernatants of HEK293 cells transfected with indicated constructs were analysed as described above.

Antibodies against SEP recognise soluble SEP protein in ELISA and are suitable
20 for diagnostic detection of SEP.

Example 20: Inhibition of SEP with antibodies

25 Antibodies against SEP were produced by immunizing rabbits with peptides PMPVQETQAPESPGENSEQAL (SEQ ID NO: 23) and PADPDGGPRPQADRK (SEQ ID NO: 24). Additionally, rats were immunized with the peptide SKLPINSTRAGM (Eurogentec, Belgium; SEQ ID NO: 25). In vitro selection of

- 61 -

human F(ab)s was performed using recombinant SEP (aa 37-510) from E.coli (Morphosys, Germany). As shown in Examples 18 and 19 the antibodies were tested in Western Blot and ELISA to show suitability for diagnostic assays. Proliferation assays with HUVEC cells were performed to determine functional activity of the antibodies (growth inhibition).

Figure 18 shows an example of growth inhibition by a rabbit anti-serum specific against SEP. HUVEC cells were incubated with indicated amounts of anti-serum and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay (as described above). Anti-SEP anti-serum reduced SEP induced growth of HUVEC cells compared to controls and is therefore suitable for therapeutic intervention of SEP activity.

The inhibition of SEP induced HUVEC proliferation by anti-SEP antibody could not only be demonstrated with polyclonal antibodies, but also with monoclonal recombinant antibody fragments. Figure 19 shows an example of growth inhibition by a human F(ab) specific against SEP generated by in vitro selection. HUVEC cells were incubated with 1 µg/ml of F(ab) and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay (see above). F(ab) reduced SEP induced growth of HUVEC cells to 71% compared to untreated cells. Recombinant antibodies and antibody fragments against SEP are therefore suitable for therapeutic intervention of SEP activity.

- 62 -

Example 21: Inhibition of SEP with peptides

Peptides derived from the extracellular domain of SEP were synthesised and tested for inhibition (competition) of SEP activity on HUVEC. The peptide
5 pWobble is a mixture of 4 peptides representing the amino acid sequence of 4 repeats within the protein sequence of SEP (N-(L/V)PSK(L/V)PT-C; SEQ ID NO: 26). The peptide p3061 is derived from this sequence (N-LPSKLPT-C; SEQ ID NO: 27).

10 Figure 20 shows an example of growth inhibition by synthetic peptides derived from the extra cellular domain of SEP. HUVEC cells were incubated with indicated amounts of peptide and supernatant of SEP transfected HEK293 cells for 5 days and proliferation of the cells was measured using the AlamarBlue Assay (as described).

15

P3061 and pWobble reduced SEP induced proliferation of HUVEC cells compared to untreated cells. Therefore peptides derived from SEP are suitable for therapeutic intervention of SEP activity.

20

Example 22: Expression of SEP in tumor cell lines

Expression levels of SEP and proliferative activity of supernatants were analysed in different tumor cell lines. For this, total RNA was extracted from tumor cell
25 lines and transcribed into cDNA. Relative expression levels were analysed by quantitative real-time PCR (QPCR) as described above. Supernatants of these tumor cell lines were transferred to HUVEC and proliferation of HUVEC was

monitored using the AlamarBlue assay as described above. Table 3 indicates expression of SEP and proliferative activity of supernatants of different tumor cell lines. The results show that increased SEP expression correlates with increased proliferative activity. To further correlate this correlation stably transfected over-expressing tumor cell lines were generated to verify the observed phenotype of enhanced HUVEC proliferation of cell supernatants of SEP overexpressing cells. For this, cells were co-transfected with a plasmid providing Neomycin resistance (pcDNA3.1, Invitrogen) and expression plasmids for SEP, SEP 1-510 or empty vector as control. Stable transfectants were selected with the Neomycin analog Geneticin (Gibco, MCF-7 800 µg/ml, PC-3 µg/ml) for 3 weeks and stable clones were isolated. Overexpression of SEP in selected clones was verified by QPCR and Western Blot analysis as described above, Table 3). Stable clones were analysed for induction of HUVEC proliferation and induction of the proangiogenic factors IL-8 and Rantes as described above. Figure 21 shows enhanced induction of HUVEC proliferation and induction of IL-8 and Rantes of stably transfected MCF-7 (A-C) and PC-3(D-F) clones compared to clones stably transfected with empty vector as control.

Table 3: Expression levels of SEP in selected tumor cell lines and corresponding proliferative activities of supernatants on HUVEC.

Cell line	Expression level of SEP	Proliferative activity on HUVEC
MCF-7	++	+ / ++
PC-3	+	+
A-549	-	-
KB-3-1	-	-

Relative expression levels of SEP were compared to relative proliferative activity of supernatants. The expression level of SEP in tumor cell lines correlates with proliferative activities of their supernatants.

Table 4: Stable transfected clones overexpressing SEP

Clone	Intergration of transgene	Over-expression of SEP (QPCR)	Over-expression of SEP (WB)	Induction of Rantes	Induction of IL-8	Proliferation of HUVEC
MCF-7 SEP 40	+	+	+	+	+/-	+
PC-3 510-1	n.d.	+	+	+	+	+

5 The indicated stable clones show overexpression of SEP and induction of IL-8 and Rantes.

10 The analysis of induction of HUVEC proliferation by stable MCF-7 clones showed that the stably SEP overexpressing clone MCF-7 SEP40 showed induction of proliferative activity on HUVEC compared to a control clone MCF-7 Vector 20 (Figure 21a). Also, we observed induction of IL-8 by the stable MCF-7 clone. For this, total RNA from MCF-7 cells stably transfected with SEP or vector control was transcribed into cDNA and relative expression of IL-8 versus G6PDH was calculated after quantitative real-time PCR (Figure 21b). Indicated is the relative induction of IL-8. The result of these experiments was that the stably SEP overexpressing clone MCF-7 SEP40 showed increased induction of IL-8 compared to a stable control clone MCF-7 Vector 20. In addition to IL-8, also induction of Rantes was observed for the stable MCF-7 clones. To show that total RNA from MCF-7 cells stably transfected with SEP or vector control was transcribed into cDNA and relative expression of Rantes versus G6PDH was calculated after quantitative real-time PCR (Figure 21c). Indicated is the relative induction of Rantes. The result of this experiment was that the stably SEP overexpressing clone MCF-7 SEP40 showed increased induction of RANTES compared to a stable control clone MCF-7 Vector 20.

- 65 -

The analysis of the induction of HUVEC proliferation by stable PC-3 clones is shown as the relative fluorescence units (RFU), given as mean value from three independent experiments in Figure 21d. Experiments were performed following the manually adapted protocol described above.

5

The clone PC-3 510-1 stably overexpressing the fragment 1-510 showed induction of proliferative activity on HUVEC compared to a stable control clone PC-3 Vector 7. We also observed induction of IL-8 by stable PC-3 clones. To show that, total RNA from PC-3 cells stably transfected with fragment 1-510 or vector control was transcribed into cDNA and relative expression of IL-8 versus G6PDH was calculated after quantitative real-time PCR (Figure 21e). Indicated is the relative induction of IL-8. The results of these analyses indicate that the clone PC-3 510-1 stably overexpressing the fragment 1-510 showed increased induction of IL-8 compared to a stable control clone PC-3 Vector 7. In addition to IL-8, we also observed induction of Rantes by stable PC-3 clones. This is consistent with the phenotype of the stable MCF-7 clones (supra). For the analysis total RNA from PC-3 cells stably transfected with fragment 1-510 or vector control was transcribed into cDNA and relative expression of Rantes versus G6PDH was calculated after quantitative real-time PCR (Figure 21f). Indicated is the relative induction of Rantes. The result of the experiment shows that the clone PC-3 510-1 stably overexpressing the fragment 1-510 showed increased induction of Rantes compared to a stable control clone PC-3 Vector 7.

20